Assessing Impact of Extended Gradient Lengths for Microflow SWATH® Acquisition

Industrialized Quantitative Proteomics using TripleTOF® 6600 System with NanoLC™ 425 System

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For quantitation of large numbers of proteins, data independent acquisition (DIA) methods[1] are currently used by many proteomics labs to perform large scale quantitative experiments on thousands of proteins in complex matrices. More recently, researchers have started to use microflow LC in order to increase robustness and throughput, while still maintaining good proteome coverage[2]. Our previous data acquired by microflow SWATH Acquisition has achieved quantification of 85% of the proteins that could be quantified using nanoflow SWATH acquisition but with an increase in throughput of 400%. This microflow LC strategy allows the analysis of up 24 sample analyses per day (~150 proteomes a week) and requires only ~ 4x more sample[1].

As a next experiment, we did a quick test to assess what gains could be achieved with a longer run time (2 hours total) and used two 15cm columns coupled together. This test provided a 17% gain in proteins quantified at a 1% peptide FDR and < 20% CVs (Figure 1). This encouraged us to do a deeper investigation to characterize the gains that would be provided by the 2 hour per sample use case.

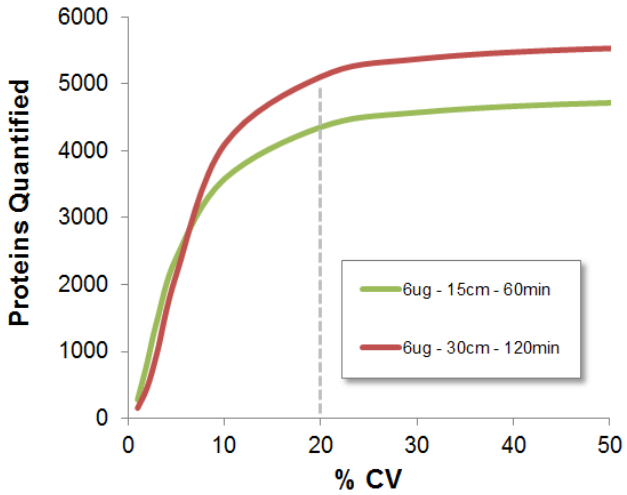

Figure 1. Protein Quantification by Microflow SWATH® Acquisition Workflow on TripleTOF® 6600 System. Microflow SWATH data for a tryptic digest of HEK cell lysate showed promising protein quantification coverage with a longer gradient and column, providing ~5100 proteins quantified in a 2 hour run time with a 6 µg sample load.

In this technical note we investigated further improvements to peptide/protein quantitation using microflow SWATH by exploring the impact of longer gradients (2 hours per sample with a 15 or 30 cm column) on 3 proteome matrices of increasing complexity.

Key Features of Microflow SWATH® Acquisition on TripleTOF® Systems

- SWATH® Acquisition combined with microflow LC provides higher throughput and robustness than nanoflow LC
  - With ~4x more sample load, a similar number of proteins are quantified using microflow as compared to nanoflow[1].
- The SCIEX NanoLC™ 425 system can be easily switched between nanoflow and microflow modes to facilitate workflow needs.
  - The trap-elute configuration provides additional robustness to the workflow by salts and other impurities

Materials and Methods

HPLC Conditions: Separations of the tryptic peptides from three samples of differing complexities (plasma, yeast and human cell lysates) were performed on a NanoLC™ 425 System (SCIEX) in microflow mode. A comparison was made between a single 0.3 x 150 mm ChromXP™ C18CL, 3 µm, 120 Å column (SCIEX), and a 30 cm column composed of two 15 cm columns connected in series with a small coupler. All tests were performed in trap-elute mode using a 0.3 x 10 mm ChromXP™ C18CL, 5 µm, 120 Å trap column (SCIEX). Tables 1 and 2 describe the two linear gradients that were compared. One is a 43 minute gradient with a 1 hour total run time and the other a 103 minute gradient with a 2 hour run time. Both used a flow rate of 5 µL/min and the column was maintained at 35 °C. Two different amounts (6 and 10 µg) of trypsin digested protein were loaded on column. Mobile phase A was 100% water with 0.1% formic acid. Mobile phase B was 100% acetonitrile with 0.1% formic acid.

MS Conditions: MS analyses were performed using SWATH® Acquisition on a TripleTOF® 6600 System equipped with a DuoSpray™ Source and 25µm I.D. electrode (SCIEX). Variable Q1 window SWATH Acquisition methods (100 windows)[2] were built in high sensitivity MS/MS mode with Analyst® TF Software 1.7.

Data Processing: Replicate data was processed using SWATH® 2.0 Software in PeakView® Software 2.2 using the Pan Human[3], Yeast[4] and Plasma[5] libraries. Data was further processed by SWATH Replicate Analysis Template 2.0 in Excel[6]. Five technical replicates were performed for each experimental condition to determine the number of peptides/proteins, identified with peptide FDR better than 1% that could be quantitated with < 20%.

Table 1. Gradient Profile for 60 Minute Total Run Time

Time (mins)	% B
0	*3*
38	*32*
43	*40*
45	*80*
48	*80*
49	*3*
57	*3*

Table 2. Gradient Profile for 120 Minute Total Run Time

Time (mins)	% B
0	*3*
98	*35*
103	*40*
105	*80*
108	*80*
109	*3*
117	*3*

Microflow LC Reproducibility

The gradient for the 60 minute run was previously optimized[2], and here the gradient for the 120 minute run was also developed and optimized. The same gradient was used for both the 15 cm column and the 30 cm coupled column configurations. Five replicate injections were performed for each experimental condition and high reproducibility of retention time (RT) and sensitivity were observed. The overlaid XICs for replicate analysis of 10 µg trypsin digested Human K562 cell lysate using the 103 min gradient and 30 cm column is shown in Figure 2.

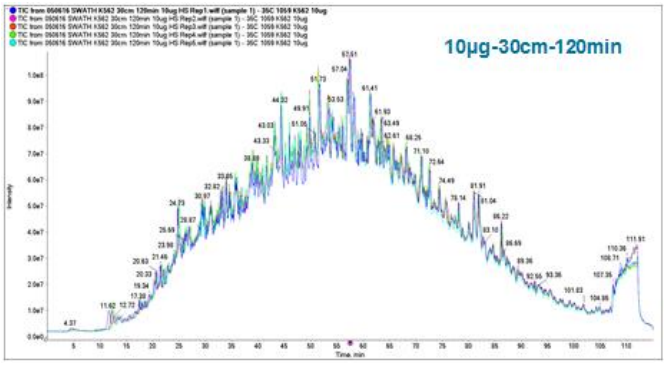

Figure 2. High Chromatographic Reproducibility with Microflow. Overlays of 5 microflow LC replicates for the 103 min gradient (30 cm column length with 10 µg load) highlight the excellent reproducibility of RT and intensity.

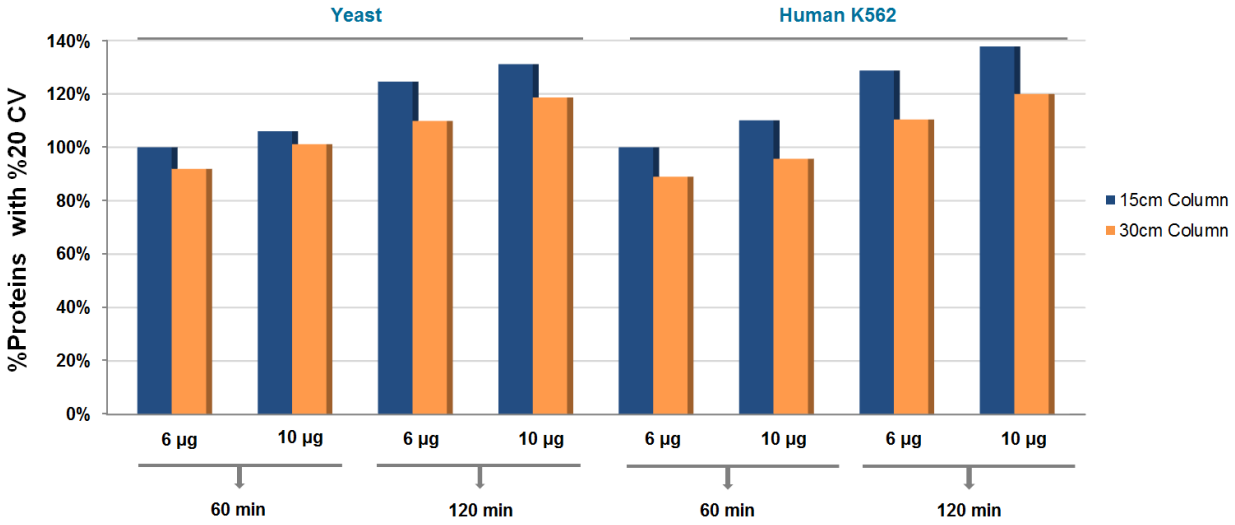

Figure 3. Effect of Gradient and Column Length on Protein Quantification. Gains of ~25% in proteins quantified (1% peptide FDR with <20% CVs) were observed for both human and yeast matrices when going from a 1 hour to a 2 hour run time. Smaller gains (5-10%) were observed when moving from a 6 to 10 µg sample load. Surprisingly, using the 30cm coupled column strategy did not provide improved results when running the longer gradient as expected, rather resulted in a small degradation in results quality.

Effect of Gradient and Column Length

The number of peptides and proteins that could be quantitated using the 1 and 2 hour total run times were compared for samples with different complexities with 6 and 10 µg loads (Figure 3). Increasing the gradient length from 43 to 103 minutes (1 to 2 hour total run time) resulted in 63% more peptides (data not shown) and ~25% more proteins with 6 µg of sample load. A similar gain was observed for the 10 µg of sample load.

We did not observe improvement with the 30cm column (2 x 15cm coupled columns) vs a 15cm column, in fact saw a small degradation. A similar result was observed for all 3 sample types. This observation was a bit surprising and will require more investigation, comparing peak widths suggested there was no significant peak broadening caused by the column coupler. But significant gains were achieved on the 15cm column suggesting this remains the desired configuration for this gradient length.

Quantification Quality using Longer Gradients

As microflow SWATH acquisition is a quantitative workflow, it is important to monitor quantitation quality constantly during method optimization. Another way to do this is to look at the % CV as a function of peak area (Figure 4). If the method is pushing the chromatography or MS too hard, then the first place this is likely to show up will be in the quantitation quality of the low abundant peptides, in the low fragment ion peak areas.

Here you can see that the curves are very similar between the experimental conditions explored.

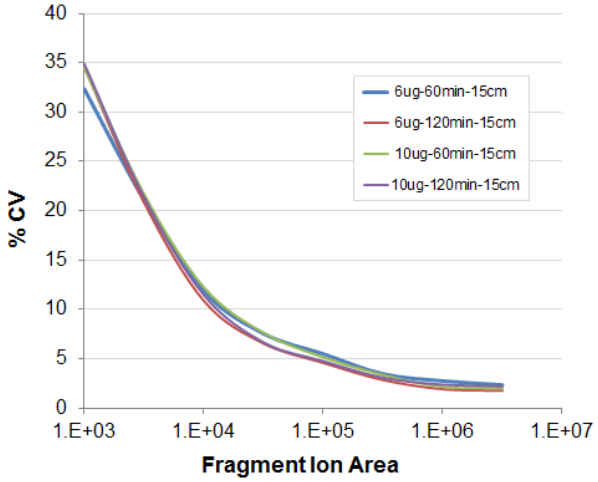

Figure 4. Assessing Quantitation Quality for the Longer Gradient Results. Here the %CV as a function of intensity is plotted for the 2 gradient lengths on the 15cm column, at the two sample loads tested. Minimal differences in reproducibility were observed even at the lowest intensity. This indicates that quality of quantitation (using reproducibility as a measure) is not degraded using the longer run times.

Quantification Improvement in Different Matrices

The number of proteins robustly quantified using microflow SWATH improved by 23-31% (Table 3) for the three different matrices tested when moving to the 2 hour per sample use case. This provides the user guidance when deciding between throughput vs quantification coverage. Table 4 provides the overall gains observed when layering on the increased sample load (6 to 10 µg). More significant improvements in peptide/protein quantification were observed with the higher load in the more complex proteomes.

Table 3. Quantification Improvement for Three Different Matrices Using the Longer 2 Hour Run Time. Here we compare the gains in protein and peptide quantitation (filtered at 1% peptide FDR and <20% CV) observed when moving from 1 to 2 hours per sample on the 15cm column, using the 10 µg sample load.

Samples	Improvements in Peptides (%)	Improvements in Proteins (%)
Plasma	*30*	*31*
Yeast	*35*	*23*
Human	*46*	*25*

Conclusions

In this work, we investigated the impact of using a longer gradient and a longer column for SWATH® acquisition experiments.

- Increasing gradient length (103 min vs 43 min) showed up to ~26% improvement in protein quantification. Best results were obtained on a 15 cm column.
- A ~8% increase in quantitation coverage was observed by increasing sample amount from 6 to 10 µg.
- When sample quantity is not a limiting factor, SWATH® Acquisition coupled with microflow chromatography provides higher throughput and robustness over nanoflow with similar peptide/protein quantification coverage.

Table 4. Total Quantification Improvement Observed. Here we compare the gains in protein and peptide quantitation (filtered at 1% peptide FDR and <20% CV) observed when moving from the 1 hour / 6 µg test to the 2 hours per sample / 10 µg test. All tests were done on the 15cm column.

Samples	Improvements in Peptides (%)	Improvements in Proteins (%)
Plasma	*31*	*26*
Yeast	*42*	*31*
Human	*76*	*38*

References

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6. SWATH replicates template 2.0 http://sciex.com/software-downloads-x2110.

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